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14. ABSTRACT

Prostate cancer may progress from an androgen-dependent to an androgen-independent state. The androgen receptor (AR) is expressed throughout this progression. We would like to understand the AR role in this progression. Using lox-Cre methodology, we have generated the mice in which AR function can be abolished in the entire animal (ARKO) or in a tissue specific manner. We will further generate mice that have AR knocked out in prostate only or in different stages to be used to study how prostate cancer progresses. Our Proposal aims follow. 1: Generate mice lacking a functional AR in prostate epithelium. 2: Generate inducible ARKO mouse line. These mice will be used to determine potential effect of androgen in absence of AR on prostate growth/maintenance. 3: Determine AR role in prostate cancer development/progression by crossing ARKO mice (from 1 and 2) with TRAMP mice. Comparing these mice will enable us to examine AR role in TRAMP induced prostate cancer and permit determination of points in prostate cancer requiring AR function. 4: Determine AR role in tumorigenicity of androgen-dependent and androgen-independent AR knockout prostate cancer cell lines. The effect of AR loss in these cells will be examined for ability to generate/promote tumors in mice.

15. SUBJECT TERMS
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Final Report Knockout AR in Prostate W81XWH-04-1-0199

•Introduction and Body (including synopses of year 1 and 2 annual report followed by third year report)

A summary of this proposal was the following statement:

While androgen receptor (AR) activity is known to be important in the development and maintenance of the normal prostate and in prostate cancer, several aspects of AR function in the prostate have not been able to be addressed until now due to a lack of appropriate animal models. We have developed mice with a floxed AR exon 2, enabling us to inactivate AR in a tissue specific or temporally specific manner. Using these mice, this proposal seeks to address several aspects of the role of AR in the prostate that could not previously be addressed with Tfm mice or by treatment with antiandrogens. The role of epithelial AR function in communication with the stroma of the fully formed prostate will be addressed in Specific **Aim 1**. This will allow an initial

determination of the androgen regulated stromal factors that may contribute to epithelial cell survival. The possible role of recently described rapid nongenomic effects of androgens in the prostate will be addressed using an inducible system to render AR non-functional in the prostate at specific developmental stages in Specific **Aim 2**. The role of AR at specific stages of prostate cancer progression will be examined in a mouse model system in Specific **Aim 3**. In Specific **Aim 4**, suppression of AR expression in prostate cancer cell lines will be examined for the effect of prostate cancer cell tumorigenicity.

- The Specific Aims and Sub Aims of the proposal were the following.

Aim 1: To generate male mice specifically lacking a functional AR in the prostate epithelium.
Aim 1a: To generate mice lacking a functional AR in the prostate.
Aim 1b: To examine the effect of loss of AR function in the prostate.
Aim 2: To generate an inducible ARKO mouse line.
Aim 2a: To generate floxAR mice that carry an inducible Cre transgene.
Aim 2b: To examine the effect of the inducible loss of AR on the development and maintenance of the prostate.
Aim 3: To determine the role of AR in prostate cancer development and progression in mice by crossing the ARKO mice generated in Specific Aims 1 and 2 with TRAMP transgenic mice.
Aim 3a: To generate mice lacking prostate specific AR function in a mouse model of prostate cancer.
Aim 3b: To determine the effect of loss of prostatic AR function in the initiation and progression of prostate cancer.
Aim 3c: To generate mice with an inducible loss of AR function in a mouse model of prostate cancer.
Aim 3d: To determine the effect of loss of AR function at specific points in the initiation and progression prostate cancer.
Aim 4: To determine the role of AR in the tumorigenicity of androgen dependent and androgen independent AR knockout prostate cancer cells.
Aim 4a: Generate prostate cancer cell lines lacking AR by somatic homologous recombination.
Aim 4b: To generate prostate cancer cell lines lacking AR by RNA interference (RNAi).
Aim 4c: To determine the effect of the removal of AR activity on tumor formation and growth in mice.

- Our proposed schedule for completion of this proposal included the following tasks.
- Task 1 (1-12 month)**:** To generate mice lacking a functional AR in the prostate.
- Task 2 (13-24 month)**:** To examine the effect of loss of AR function in the prostate.
- Task 3 (1-12 month)**:** To generate floxAR mice that carry an inducible Cre transgene.
- Task 4 (13-24 month)**:** To examine effect of inducible loss of AR on the development and maintenance of the prostate.
- Task 5 (1-12 month)**:** To generate mice lacking prostate specific AR function in a mouse model of prostate cancer.
- Task 6 (13-24 month)**:** Determine effect of loss of prostatic AR function in initiation and progression of prostate cancer.
- Task 7 (1-13 month)**:** To generate mice with an inducible loss of AR function in a mouse model of prostate cancer.
- Task 8 (13-36 month)**:** To determine effect of loss of AR function at specific points in initiation and progression of prostate cancer.
- Task 9 (1-18 month)**:** To generate prostate cancer cell lines lacking AR by somatic homologous recombination.
- Task 10 (1-12 month)**:** To generate prostate cancer cell lines lacking AR by RNA interference (RNAi).
- Task 11(19-36 month)**:** To determine the effect of the removal of AR activity on tumor formation and growth in mice.

•Synopsis of Body of 1st report Oct-05

Previous studies using prostate tissue recombination demonstrated that androgen receptor (AR) in stromal cells, but not in epithelium plays essential role for the prostate development. and androgen-induced paracrine growth factor signals from the mesenchyme might induce and pattern the adjacent epithelial development. Yet so far, no single growth factor has been identified that can completely restore the stromal AR function, suggesting multiple growth factors, including those in the epithelium might all contribute to the prostate development.

Most of the epithelial-stromal recombination studies discontinue experiments at 4th week, and therefore prevent further study of the epithelial AR roles in the adult prostate development. Mice lacking both stromal and epithelial ARs fail to develop prostate, which also prohibits our study of AR roles in prostate development. We generated prostate epithelium-specific AR knockout (pes-ARKO) mice, representing the first available *in vivo* animal model to study the influence of loss of AR in adult prostate epithelia.

The results and description of 2 of the figures were written in the 1st annual report but deleted here in the final report as the entire manuscript has been attached.

•Key Accomplishment List from 1st Annual report

- Task 1 (1-12 month)**:** We generated mice lacking a functional AR in the prostate epithelium (pes-ARKO).
- Task 3 (1-12 month)**:** We generated floxAR mice that carry an inducible Cre transgene (ind-ARKO).
- Task 5 (1-12 month)**:** We demonstrated that our pes-ARKO mice lack prostate specific AR function (Fig. 1).

•Task 7 (1-13 month)**:** To generate mice with an inducible loss of AR function in a mouse model of prostate cancer (ind-pes-ARKO). This task has been initiated but the proper genotypes are slowly being raised and the inducible model takes time before it can been proven and will be reported in the next annual report.
•Task 9 (1-18 month)**:** To generate prostate cancer cell lines lacking AR by somatic homologous recombination. This task was initiated, but no statistically significant results during the reporting period.
•Task 10 (1-12 month)**:** To generate prostate cancer cell lines lacking AR by RNA interference (RNAi). This task was initiated, but no statistically significant results during the reporting period.

•Reportable Outcomes from 1st Annual report:
• flox AR mice
• pes-ARKO mouse model

•A synopsis of the Body of the 2nd annual report most important results follows, again results of figures presented are removed as the manuscript has been attached.

Task 2 (13-24 month)**:** To examine the effect of loss of AR function in the prostate. Pes-ARKO mice AR gene deletion within prostate epithelium was verified by genotyping, as well as the specificity of recombination in several key organs by RT-PCR with deletion of AR exon 2 was confirmed by the detection of truncated transcripts present within the ventral prostate and dorsal-lateral prostate of pes-ARKO mice only. There were no differences in external characteristics, including genital-anal distances or in internal urogenital organs between WT and pes-ARKO mice. In contrast, pes-ARKO mice had significantly larger ventral prostates at week 24 and neither the dorso-lateral prostate nor anterior prostate within pes-ARKO mice significantly changed in size.
Task 6 (13-24 month)**:** To determine the effect of loss of prostatic AR function in the initiation and progression of prostate cancer. We confirmed the progressive loss of AR by immunohistochemistry. AR protein localized to epithelial nuclei slowly decreased with age in pes-ARKO mice compared to WT littermates. By week 24 epithelial AR detection was rare. To evaluate epithelial AR signaling, probasin intensity was similar in pes-ARKO and WT littermates until 12 weeks of age, when the reduced level in pes-ARKO samples compared to WT approached significance. By week 24 and thereafter, this difference was significant. To determine if pes-ARKO mice contain abnormalities other than enlarged ventral prostates, we evaluated fertility. We found that there were no significant differences in litter-size when either WT or pes-ARKO males were mated to WT females. To rule out the possibility of altered ventral prostate size might be due to circulating androgen levels, we measured serum testosterone levels by ELISA. We observed no difference between the WT and pes-ARKO males at 12 or 24 weeks of age. Together, results demonstrate an effective deletion of the AR that is confined to the prostatic epithelium and that consequences of the AR gene deletion appears to be restricted to the prostate and are without the influence of serum testosterone.

•Key Research Accomplishments and Reportable Outcomes from 2nd Annual report:
•Task 1, 3, and 5 have been completed and we continue to generate more mice for experiments in other Aims and tasks.
•Task 2 (13-24): To examine the effect of loss of AR function in the prostate. Results are in themanuscript published.
•Task 4 (13-24 month)**:** To examine the effect of the inducible loss of AR on the development and maintenance of the prostate. Due to the low breeding ratios we have not been able to complete the experiments yet.
•Task 6 (13-24 month)**:** To determine the effect of loss of prostatic AR function in the initiation and progression of prostate cancer. Several experiments have been completed or are in the process of being completed. Results are in the Figure presented.
•Task 7 (1-13 month)**:** To generate mice with an inducible loss of AR function in a mouse model of prostate cancer (ind-pes-ARKO). This task has been initiated, the proper genotypes have been produced in small numbers.
•Task 8 (13-36 month)**:** To determine the effect of loss of AR function at specific points in the initiation and progression of prostate cancer. Several experiments have been completed or are in the process of being completed. Results are in the Figures presented. The experiments reported for Task 6 are part of the experiments planned to complete this task.
•Task 9 (1-18 month)**:** To generate prostate cancer cell lines lacking AR by somatic homologous recombination. These cell lines have been more difficult to develop than expected, but are in the final stages of being tested and experiment results will be reported in next progress report.
•Task 10 (1-12 month)**:** To generate prostate cancer cell lines lacking AR by RNA interference (RNAi). This task was initiated, but no statistically significant results during the reporting period due to difficulty in development of the cell lines.
Task 11(19-36 month)**:** To determine the effect of the removal of AR activity on tumor formation and growth in mice. Several experiments have been completed or are in the process of being completed. Results are in the Figure presented at the end of the report following References and Appendices.

•Reportable Outcomes from 2nd report:
• flox AR mice
• pes-ARKO mouse model

•**Conclusions:** The first 2 paragraphs are synopsis of the conclusions from the annual reports
•Conclusion from 1st year report

We have made very promising progress in this first year even though there is only the one figure presented in this annual report. The inducible mouse model, and other cell lines that are in production are very time consuming and difficult to produce, but are being produced in enough numbers for further experiment within the second year of the grant. All of the initiated tasks will allow us to continue with the other tasks towards completion of the entire project within the 3 years.

•Conclusions: from 2nd year report

A key signature of the adult normal prostate gland is the lack of proliferation even in the presence of growth stimulating androgens. This is in contrast to benign prostate hyperplasia and prostate cancer, in which epithelial cells acquire the ability to proliferate. First we report that in mature prostatic epithelium, AR is critical for maintaining differentiated phenotype and overall homeostasis of the gland. Moreover, selective removal of epithelial AR signaling stimulates mitogenesis of the otherwise growth quiescent prostate. Normal prostate growth may require delicate temporal and spatial balance between the proliferative role of stromal AR and the growth-suppressive role of epithelial AR. Our findings recast the role of androgen/AR signaling within the prostate, and question the current therapeutic strategy for prostate disease, which relies solely and indiscriminately on antagonizing stromal ARs to prevent proliferation without considering epithelial AR's suppressive roles.

•3rd year report-final year

•Body of final year report.

During the last year of the project we were able to continue and complete the tasks started in the first 2 years and continued to work on the tasks stated for the 3rd year.

•Task 4 (13-24 month)**:** To examine the effect of the inducible loss of AR on the development and maintenance of the prostate. Due to the low breeding ratios we have not been able to complete the experiments yet. This task, however was not able to be completed as yet even though we have developed a few inducible mice the experiments are yet to be finished to give statistically significant results. They will be continued using other funds available to the lab.

•Task 8 (13-36 month)**:** To determine the effect of loss of AR function at specific points in the initiation and progression of prostate cancer. Several experiments have been completed and results are in the manuscript published.

•Task 11(19-36 month)**:** To determine the effect of the removal of AR activity on tumor formation and growth in mice. Several experiments have been completed and results are in the manuscript published

The paper below was published in *PNAS* and has been attached at the end of the report.

Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor

Chun-Te Wu, Saleh Altuwaijri , William A. Ricke, Shu-Pin Huang, Shuyuan Yeh, Caixia Zhang, Yuanjie Niu, Meng-Ying Tsai, Kuo-Pao Lai, Chawnshang Chang *PNAS.* 104(31):12679–12684

A brief Abstract of the paper follows:

Developmental studies have established that ductal morphogenesis, epithelial cytodifferentiation, and proliferation/apoptosis are regulated by androgens acting through stromal androgen receptor (AR). Here we found mice lacking epithelial AR within the mature prostate (pes-ARKO) developed prostate tissue that was less differentiated and hyper-proliferative relative to wild-type littermates. Circulating levels of testosterone, external genitalia, or fertility were not altered in pes-ARKO mice. A significant increase in BrdU-positive epithelial was observed in ventral and dorsal-lateral prostates of pes-ARKO mice. Differentiation markers probasin, PSP-94, and Nkx3.1 were significantly decreased and epithelial sloughing and luminal cell apoptosis increased from 6-32 weeks of age in pes-ARKO mice. Gain of function occurred by crossing pes-ARKO to the T857A transgenic mice containing constitutively activated AR. In T857A-pes-ARKO mice prostates were of normal size, contained glandular infoldings, maintained high secretory epithelium, and the appropriate prostatic epithelial proliferation was restored. Collectively these results suggest that prostatic epithelial AR plays an important role in the homeostasis of the prostate gland and support the hypothesis that epithelial AR controls prostate growth by suppressing epithelial-proliferation in the mature gland.

•Key Research Accomplishments See above from 1st and 2nd annual reports and the following from the manuscript.

•Loss of differentiated glandular structure in pes-ARKO mice
•Loss of epithelial AR decreases androgen regulated gene expression.
•Mature prostate growth is increased in pes-ARKO mice
•Sloughing and apoptosis of epithelia in the prostate of pes-ARKO mice.
•Restoring functional AR via knock-in of T857A-AR restores pes-ARKO to a normal prostate phenotype

•Reportable Outcomes:

- flox AR mice
- pes-ARKO mouse model
- T857A-pes-ARKO mice

•Publications:

•The manuscript results that both the 1st and 2nd annual report refers to, which was completed during the final year of the project has been published in PNAS.

Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor
Chun-Te Wu, Saleh Altuwaijri , William A. Ricke, Shu-Pin Huang, Shuyuan Yeh, Caixia Zhang, Yuanjie Niu, Meng-Ying Tsai, Kuo-Pao Lai, Chawnshang Chang, *PNAS.* 104(31):12679–12684

- **Conclusion; Please see synopsis of 1st ad 2nd year's report as well as the conclusion below taken partly from the manuscript and partly from the preceeding years conclusions. In general most of the tasks of the proposal were completed resulting in positive results for the proof of the proposals hypotheses.**

A key signature of the adult normal prostate gland is the lack of proliferation even in the presence of growth stimulating androgens. This is in contrast to benign prostate hyperplasia and prostate cancer, in which epithelial cells acquire the ability to proliferate. Here we show 2 seminal findings in the area of cell biology. We found *in vivo* and *in situ* that in mature prostatic epithelium, AR is critical for maintaining differentiated phenotype and overall homeostasis of the gland, and that selective removal of AR signaling in luminal epithelial cells stimulates mitogenesis of the otherwise growth quiescent adult prostate. These data support the hypothesis that AR in differentiated prostatic epithelium maintains homeostasis via induction of epithelial growth suppressors (or decreased production of growth-stimulatory factors) that may indirectly mediate stromal factors or act directly on putative AR-negative progenitor (i.e. transit amplifying or intermediate) cells, thereby inhibiting epithelial proliferation. The mechanisms by which AR mediates these processes may be multi-factorial, most likely involving paracrine factors. Normal prostate growth may require delicate temporal and spatial balance between the proliferative role of stromal AR and the growth-suppressive role of epithelial AR. Our findings recast the role of androgen/AR signaling within the prostate, and suggest that future chemoprevention strategies may need to target stromal-AR-mediated-factors rather that epithelial-AR-mediated-factors to prevent early stages of carcinogenesis

- **Personnel supported by this grant**
- Chawnshang Chang
- Saleh Altuwaijri
- Hsin-Chiu Ho
- Gong-Hui Li
- Tongzu Liu
- Huei-Ju Ting
- Shian-Jang Yan.

- **References:** None

- **Appendix:** one manuscript published.

Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor
Chun-Te Wu, Saleh Altuwaijri , William A. Ricke, Shu-Pin Huang, Shuyuan Yeh, Caixia Zhang, Yuanjie Niu, Meng-Ying Tsai, Kuo-Pao Lai, Chawnshang Chang *PNAS.* 104(31):12679–12684

Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor

Chun-Te Wu*†, Saleh Altuwaijri*‡, William A. Ricke*, Shu-Pin Huang*§, Shuyuan Yeh*, Caixia Zhang*, Yuanjie Niu*, Meng-Ying Tsai*†, Chawnshang Chang*¶

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AQ: A

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Developmental studies in the prostate have established that ductal morphogenesis, epithelial cytodifferentiation, and proliferation/apoptosis are regulated by androgens acting through stromal androgen receptor (AR). Here, we found mice lacking epithelial AR within the mature prostate (pes-ARKO) developed prostate tissue that was less differentiated and hyperproliferative relative to WT littermates. Epithelial AR protein was significantly decreased in 6-week-old mice and was nearly absent by ≥24 weeks of age. Circulating levels of testosterone, external genitalia, or fertility were not altered in pes-ARKO mice. A significant ($P < 0.05$) increase in bromo-deoxyuridine-positive epithelia was observed in ventral and dorsal-lateral prostates of pes-ARKO mice at 24 weeks of age. Less differentiation was observed as indicated by decreased epithelial height and glandular infolding through 24 weeks of age and differentiation markers probasin, PSP-94, and Nkx3.1 were significantly decreased and epithelial sloughing and luminal cell apoptosis increased from 6–32 weeks of age in pes-ARKO mice. Gain of function occurred by crossing pes-ARKO to the T857A transgenic mice containing constitutively activated AR. In T857A-pes-ARKO mice prostates were of normal size, contained glandular infoldings, maintained high secretory epithelium, and the appropriate prostatic epithelial proliferation was restored. Collectively, these results suggest that prostatic epithelial AR plays an important role in the homeostasis of the prostate gland. These data support the hypothesis that epithelial AR controls prostate growth by suppressing epithelial proliferation in the mature gland.

AQ: B, C, D

testosterone

Fn1

Androgens and epithelial-mesenchymal interactions are necessary for prostate growth and development. Androgen signaling occurs through the androgen receptor (AR) (1, 2), which is found in both stroma and epithelium within the prostate. Mice lacking a functional androgen/AR signaling fail to develop normal prostate glands (3, 4). Pioneering studies on normal prostatic development showed that stromal, but not epithelial AR, is essential for specification of epithelial cell identity, bud formation, ductal branching, proliferation, and apoptosis (5, 6). In contrast, experimental evidence from study of anaplastic prostate cancer cell lines has led to the idea that epithelial AR, when activated by androgen, increases cellular proliferation (7–9). This notion is the central premise for androgen ablation therapy, a key treatment for advanced or metastatic prostate cancer. Although many studies demonstrate that stromal, but not epithelial, AR mediates key events during normal prostatic development (5, 6), these studies were typically evaluated over short periods of time, thus recapitulation of events that may take months to manifest a phenotype were not examined. To date, there are no models or techniques that effectively evaluate the role of epithelial AR *in situ* or *in vivo* within the mature adult and aging prostate. In addition, it is unclear as to why the mature prostate maintains homeostasis without active proliferation in an androgenic milieu.

In the adult prostate, androgen deprivation leads to luminal cell apoptosis and dedifferentiation, resulting in an increased proportion of basal cells to luminal cells (10, 11). In normal prostate, benign prostatic hyperplasia, and prostate cancer, androgen deprivation decreases growth, increases apoptosis, and reduces tumor volume and is thought to be acting on the epithelial or carcinoma cells. Often, however, the effect is temporary and after removal of androgens, abnormal epithelial cells persist and inevitably grow independent of hormonal stimulus. Every year, >30,000 men die and many more suffer from this enigmatic process. To better understand the role of androgens/AR signaling in normal and malignant prostate epithelial function, it is imperative to evaluate gain and loss of function of AR within the prostatic epithelia. Here, we report the generation of the conditional knockout AR (pes-ARKO) mice that lack AR only in prostate epithelia.

BIOCHEMISTRY

Results and Discussion

Generation and Characterization of pes-ARKO Mice. Pes-ARKO mice contain a prostate epithelial specific promoter (12) driving cre-recombinase in floxed AR mice (3). Expression of the probasin promoter transgene within the epithelium has been reported to be increasingly expressed from 2–7 weeks and sustained expression within the luminal cells is observed throughout life (13). To verify AR gene deletion within prostate epithelium of pes-ARKO mice, candidate mice were genotyped for probasin-cre transgene and conditional flox-AR allele (Fig. 1*a*). We also evaluated the specificity of recombination in several key organs by RT-PCR, using primers directed toward exons 1 and 3 of the AR gene. Deletion of AR exon 2 was confirmed by the detection of truncated transcripts via RT-PCR present within the VP and dorsal-lateral prostate (DLP) of pes-ARKO mice only (Fig. 1*b*). The lobe specific expression is consistent with the probasin promoter transgene driven expression in other models (14). No other tissues in WT or pes-ARKO mice contained truncated forms of AR DNA.

F1

There were no differences in external characteristics, including anogenital distances between WT and pes-ARKO mice (Fig.

Author contributions: C.-T.W., S.A., and W.A.R. contributed equally to this work; C.C. designed research; C.-T.W., S.A., W.A.R., S.-P.H., S.Y., C.Z., Y.N., and C.C. performed research; C.Z. and M.-Y.T. analyzed data; and W.A.R. wrote the paper.

AQ: N

The authors declare no conflict of interest.

Abbreviations: AR, androgen receptor; DLP, dorsal-lateral prostates; pes-ARKO, conditional knockout androgen receptor; VP, ventral prostate.

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ZSI

This article contains supporting information online at www.pnas.org/cgi/content/full/0704940104/DC1.

Cadmus: 7142

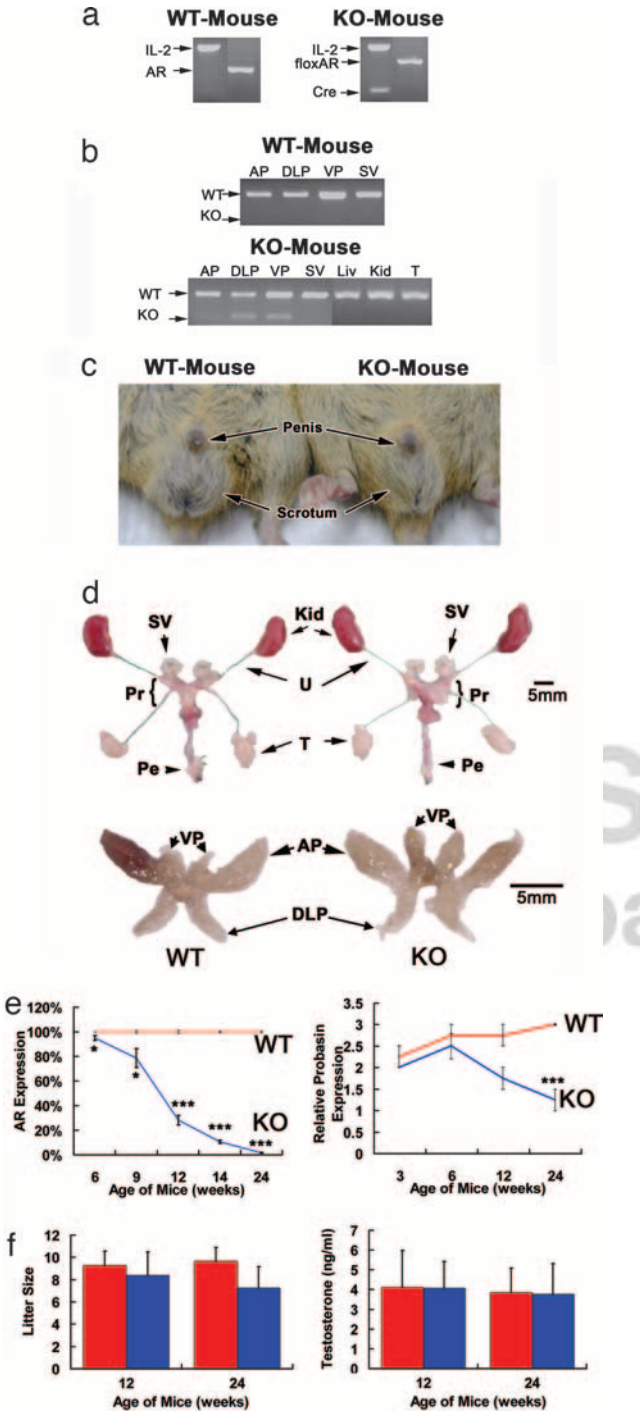

Fig. 1. Characterization of pes-ARKO mice. (*a*) Genotypes of WT (*Left*, WT) and pes-ARKO mice (*Right*, KO). The presence of transgenes cre (110 bp) and floxAR (540 bp) were observed only within pes-ARKO mice. Only WT mice have WT AR gene. IL-2 RNA is present in both mice and serves as internal positive control. (*b*) Evaluation of AR and floxed AR using RT-PCR was performed by using primers that span exon 1 through exon 3 of the AR gene. This result shows one band for WT AR (305 bp) in WT mice. In pes-ARKO mice, which lack exon 2, both WT band (WT AR is present in stroma) and the knockout band (153 bp; deletion of exon 2) appear within DLP and VP, but only the WT band appears in other tissues. (*c* and *d*) Evaluation of external (*c*) and internal (*d*) organs demonstrated no differences between strains, except for the larger VP size in pes-ARKO mice. (*e*) Assessment of protein expression of AR (*Left*) and probasin (*Right*) in VPs of WT vs. pes-ARKO mice demonstrate a decrease over time in pes-ARKO mice VPs but not in WT mice VPs. (*f*) To assess fertility, pups per litter from WT female × WT males (red bar) or × pes-ARKO (blue bar) males were compared and found to be not significantly different (*Left*). Serum testosterone levels (*Right*) were similar in WT (red bar) and pes-ARKO (blue bar) male mice at weeks 12 and 24 and thus the changes in prostatic phenotypes are not likely to be due to changes in circulating androgens. SV, seminal vesicle; Kid, kidney; U, ureter; AP, anterior prostate; Pr, all lobes of prostate; T, testes; Pe, glans penis. *, $P < 0.05$; ***, $P < 0.001$.

1*c*). The internal urogenital organs also showed no differences between WT and pes-ARKO mice (Fig. 1*d Upper*). In contrast, pes-ARKO mice had substantially larger VPs at week 24 (Fig. 1*d Lower Right*). Neither the DLP nor anterior prostate within pes-ARKO mice significantly changed in size.

We confirmed the progressive loss of AR by immunohistochemistry. A labeling index for epithelial AR was determined and tabulated (Fig. 1*e Left*). AR protein localized in epithelial nuclei slowly decreased with age in pes-ARKO mice compared with WT litter mates. By week 24, epithelial AR detection was rare. To indirectly evaluate epithelial AR signaling, we quantified staining intensity of probasin, an androgen regulated protein in mature animals. Probasin intensity was similar in pes-ARKO and WT littermates until 12 weeks of age, when the reduced level in pes-ARKO samples compared with WT approached significance ($P = 0.057$). By week 24 and thereafter, this difference was significant ($P = 0.027$) (Fig. 1*e Right*). These data suggest that probasin expression is normal before significant loss of AR and epithelial AR decline precedes the loss of an AR-dependent secreted protein, thus confirming earlier studies by Donjacour and colleagues (15).

To determine whether pes-ARKO mice contain abnormalities other than enlarged VPs, we evaluated fertility. We found that there were no significant differences in litter-size when either WT or pes-ARKO males were mated to WT females (Fig. 1*f*). To rule out the possibility that elevated VP size might be due to circulating androgen levels, we measured serum testosterone levels by ELISA. We observed little difference between the WT and pes-ARKO males at 12 or 24 weeks of age (Fig. 1*f Right*). Together, results shown in Fig. 1 demonstrate an effective time-dependent depletion of the AR that is confined to the prostatic epithelium and that consequences of the AR gene deletion appear to be restricted to the prostate and are without the influence of serum testosterone.

Loss of Differentiated Glandular Structure in pes-ARKO Mice. The histomorphology of prostates was checked weekly from 2 through 6 weeks of age and then biweekly thereafter until 32 weeks for the anterior, dorsal-lateral, and VPs. Early on, the pes-ARKO glands looked normal, showing considerable prostatic budding, and differentiation into tall, columnar glandular epithelium (Fig. 2). In pes-ARKO mice at 9 weeks of age, some ducts within the VP contained epithelial cells that were shorter in height or low cuboidal as compared with taller or columnar epithelia from WT littermates. Concurrent with loss of prostatic epithelial morphology was the loss of glandular infolding in the pes-ARKO mice (Fig. 2*a*). These changes in histomorphology increased over time, until, at 24 weeks (and later), nearly all ducts contained a dedifferentiated epithelium of low height and lack of glandular infolding (Fig. 2*a*).

In pes-ARKO mice at 14 weeks of age, an increased number of cells were found as detached layers within the prostatic lumen (Fig. 2*e*). In ducts where histological dedifferentiation appeared, normal glandular infolding could be observed (Fig. 2*b*) as well as a range of infoldings with constrictions at their base (Fig. 2 *c* and *d*). As the infoldings became narrower at their base, cells appeared to have lost polarity, as observed by nuclei moving from basal to apical location. Ultimately glandular infoldings were lost, and apparently sloughed into the prostatic lumen (Fig. 2*e*).

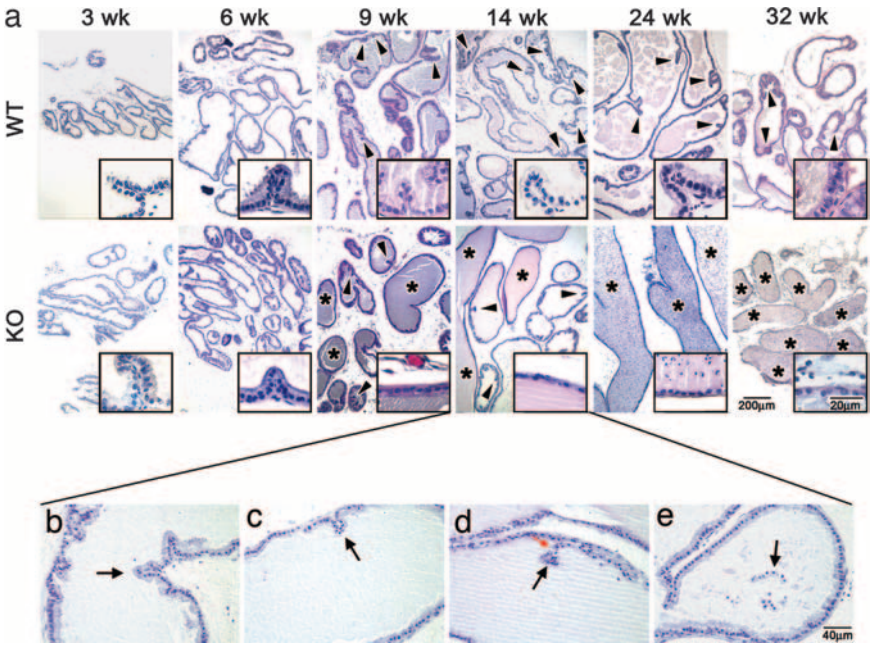

Fig. 2. Histomorphological changes in the VP of pes-ARKO mice. (*a*) H&E staining of VP of WT mice show glandular infoldings (arrowheads) and tall secretory epithelium through week 32. In pes-ARKO (KO) littermates, we saw these features only at weeks 3 and 6. In week 9 pes-ARKO mice, some VP ducts lose glandular infoldings (*) and have short, poorly differentiated epithelial cells compared with WT littermates. The change in epithelia is evident in ≈50% of ducts within the VP of week 14 pes-ARKO mice. At week 24 (and older), in pes-ARKO mice nearly all glandular infolding and high secretory cells are lost and the enlarged ducts have many sloughed epithelial cells, fragmented nuclei, and immune cells. By week, 32 pes-ARKO mice lack glandular infolding and have squat epithelial cells. (Magnification: ×100; *Inset*, ×400.) (*b*–*e*) At week 14, in pes-ARKO mice, the VP continues to lose glandular infolding. Layers of sloughed epithelial cells are abundant in the prostate lumen. (*b*) In week 14 pes-ARKO mice, some glandular infoldings (arrow) are present. (*c*–*e*) In pes-ARKO mice, infoldings (arrow) become smaller and shorter (*c*) and infoldings (arrow) constrict (red arrow) at their base (*d*). Ultimately (*e*), putative glandular infoldings (arrow) are lost and sloughed luminal cells appear within the lumen.

BIOCHEMISTRY

Loss of Epithelial AR Decreases Androgen Regulated Gene Expression. Ventral prostates (VPs) from WT and pes-ARKO mice of different ages were stained for AR and androgen regulated probasin [supporting information (SI) Fig. 5]. At week 3, AR staining in both epithelial and stromal cells was evident in the two strains. At week 6 the pes-ARKO prostates start to have noticeably weaker epithelial AR staining and by week 24 AR staining in the epithelium was undetectable. There were also a small percentage of epithelial cells within the DLP of pes-ARKO mice that lacked AR protein; however, nearly all luminal cells of the anterior prostate were positive for AR. The decline of probasin staining in VP epithelium of the pes-ARKO mice lagged behind that of AR, but was also gone by week 24 (SI Fig. 5). Importantly, stromal AR was seen at all stages evaluated.

Probasin, Nkx3.1, and prostatic secretory protein-94 (PSP94) are three prostate-specific proteins known to be transcriptionally regulated by androgens (16, 17). However, it is unknown whether stromal AR or epithelial AR is responsible for their expression. To evaluate loss of epithelial AR signaling on downstream gene expression, we performed quantitative PCR on VP RNA from WT and pes-ARKO mice at weeks 6, 12, 18, and 32. In pes-ARKO VPs, transcriptional down-regulation of probasin and PSP94 were observed by week 18 and remained lower through the final time point at week 32 (Fig. 3*a*). Nkx3.1 is a transcription factor that governs prostate morphogenesis and patterning (18) and is a marker of tumor initiation and progression (19). Nkx3.1 gene expression is significantly ($P < 0.05$) decreased by 12 weeks and remained low through 32 weeks (Fig. 3*a*). The decreased expression of Nkx3.1 coincides with the marked loss of glandular infolding. Thus, AR signaling is significantly decreased within prostate epithelia by week 12, and that epithelial AR is the major transcriptional regulator of these genes.

Although epithelial AR is significantly decreased by 6 weeks, androgen regulated factors (probasin, PSP94, Nkx3.1) have a delayed response in their reduction in gene expression. It is unclear what causes this delay, which might be due to an as-yet undescribed mechanism that is epithelial AR-independent, for example, stromal AR dependency. These data agree with the report suggesting that epithelial AR governs secretory protein expression (15) and suggest that loss of epithelial AR signaling leads to both biochemical and structural dedifferentiation of the mature prostate.

Mature Prostate Epithelial Cell Proliferation Is Increased in pes-ARKO Mice. Tissue growth occurs through hypertrophy and/or hyperplasia and is balanced by cell death. The normal adult prostate is growth-quiescent, whereas in prostatic disease organ size and epithelial proliferation increases. At 24 weeks of age, VPs of the pes-ARKO mice were larger than those of their WT littermates. As noted in our histological examination of VPs from pes-ARKO mice, epithelial cells shrunk in size, suggesting that VP enlargement was not due to hypertrophy (Fig. 2*a*, 14–32 wk). To check for VP proliferation, we evaluated BrdU incorporation. Up to week 14, in prepubescent and mature prostates regardless of WT or knockout genotype, BrdU incorporation was not different and was primarily localized to epithelial cell nuclei (Fig. 3*b*). However, by week 24, concurrent with nearly complete loss of epithelial AR, BrdU incorporation was significantly higher in prostatic epithelium of pes-ARKO mice than in WT littermates (Fig. 3 *b*–*c* and SI Fig. 6 *c* and *d*), which was confirmed to have elevated proliferating cell nuclear antigen (PCNA) within prostatic epithelium of pes-ARKO (data not shown). The observed significant increase ($P < 0.05$) in proliferation was evident in all prostatic lobes except for the anterior prostate. Although a significant increase was observed in DLP and VP, only the VP

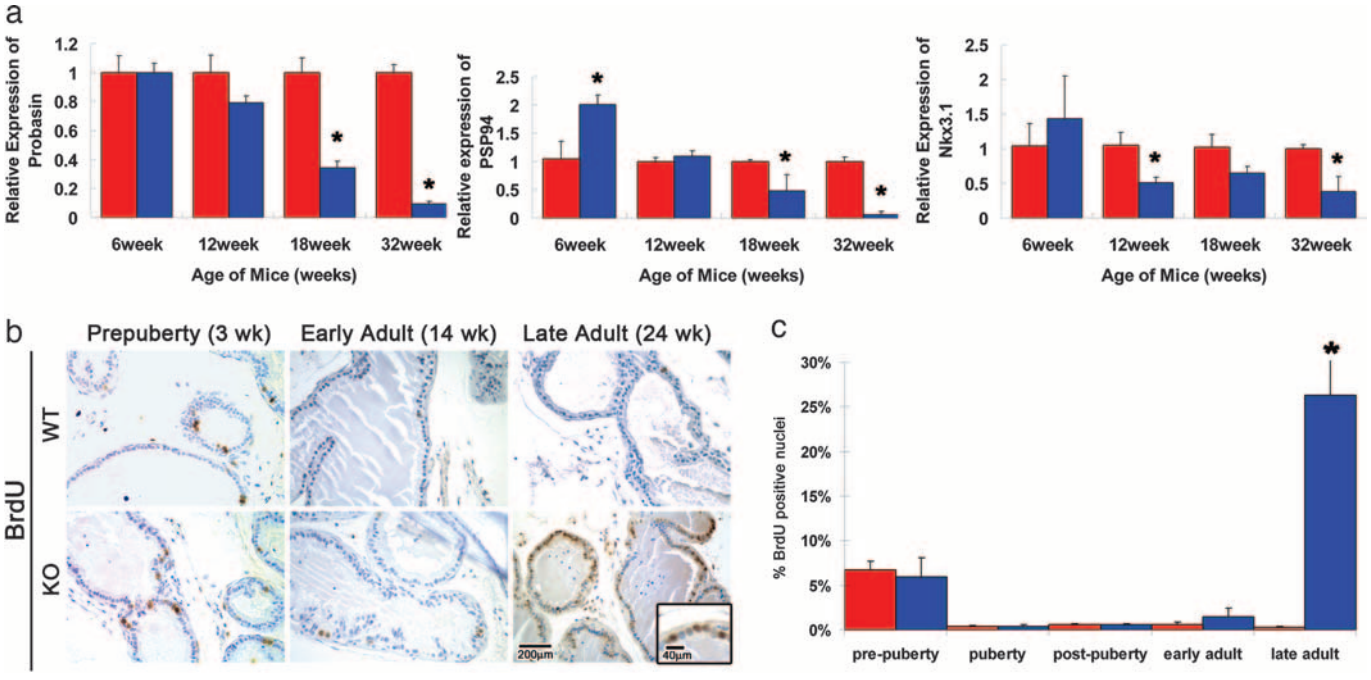

Fig. 3. Loss of epithelial androgen receptor leads to loss of androgen regulated protein and gene expression and increased proliferation. (*a*) Androgen regulated gene transcription decreases as epithelial ARs are lost. Quantitative PCR was done on VPs from WT (red bar) and pes-ARKO (blue bar) mice. Androgen regulated genes probasin, PSP94, and Nkx3.1 are all down-regulated in pes-ARKO prostates compared with WT prostates. (Magnification: ×100; *Inset*, ×400.) (*b*) VPs were collected from WT (red bar) and pes-ARKO (blue bar) mice during different stages and analyzed for proliferation. Proliferation, as determined by BrdU positive nuclei primarily occurs in epithelial cells at all stages evaluated. (*c*) Epithelial cell proliferation is significantly ($P < 0.01$) higher in pes-ARKO than WT littermates. Prepuberty (2–3 weeks), puberty (4–6 weeks), postpuberty (7–8 weeks), early adult (9–22 weeks), late adult (24–32 weeks). *, $P < 0.05$.

increased in size. The reason for the increased VP size is unclear. Because prostate epithelia of the VP had a substantially higher BrdU labeling-index relative to DLP, it may be that increased cellular proliferation of the VP lead to its increased size, whereas a smaller proliferation rate observed in the DLP did not lead to a significant increase in size. It is possible that loss of AR might increase epithelial proliferation through an Nkx3.1 mediated pathway, because hyperplasia has been observed in Nkx3.1 deficient mice. Although in pes-ARKO mice, increased proliferation was observed through 56 and 80 weeks of age ($n = 2$; data not shown), no carcinoma *in situ* was observed. It has been widely interpreted that proliferation of normal prostatic epithelial cells (7, 8) and hence carcinoma cells (20, 21) are induced to proliferate by androgens acting directly through epithelial AR. However, others have demonstrated that androgens acting directly on epithelial AR might not directly regulate proliferation (15, 22, 23). Maintenance of epithelial proliferation in aged adult prostate lacking epithelial AR suggests that epithelial androgen/AR signaling might induce production of proliferation suppressors within luminal epithelial cells. Such epithelial derived proliferation suppressors might act directly to suppress epithelial proliferation or may act indirectly to regulate epithelial or stromal production of growth factors, which in turn regulate epithelial proliferation. The lack of AR within the epithelium of pes-ARKO mice spatially recapitulates what is observed in fetal and neonatal prostate development in that AR is present only within the stroma and not in the epithelium (24, 25). Interestingly, during this time of development, epithelial proliferation is high (26, 27). The results presented here represent a concept in prostate biology in which epithelial AR is capable of controlling epithelial mitogenesis by acting as a suppressor of epithelial proliferation in the mature prostate.

Using immunocytochemical markers for basal and luminal epithelial cells, we then determined that, as the pes-ARKO animals matured, the p63-positive basal epithelial cell population increased during puberty and then remained elevated, while the cytokeratins-8 and -18-positive luminal epithelial cell population declined. In contrast, in the WT animals, the basal cell number declined with age, whereas the luminal cell (CK8/18-positive cells) population remained stable (SI Fig. 7). ZSI

To evaluate cell death, we used histological and TUNEL staining in VPs from pes-ARKO mice (SI Fig. 7*b*). We saw little ZSI apoptosis or necrosis in the prostatic epithelium of pes-ARKO mice. In the lumen, however, we saw considerable cell death in CK8-positive cells, but not CK5-positive cells (SI Fig. 3). These ZSI data demonstrate that lack of epithelial AR/AR signaling leads to sloughing of epithelial cells into the lumen (Fig. 2 *b–d* and SI ZSI Fig. 6*a*) and ultimately epithelial cell death (SI Fig. 6*b*). ZSI

To evaluate which population of epithelial cells increased over time in pes-ARKO mice, we identified each cell type, using cell specific markers. Basal and luminal cells were identified histochemically, using antibodies directed toward CK5 or p63 (28, 29) and cytokeratins-8 and -18 (30), respectively. Expression of p63 is critical for maintaining the progenitor-basal cell population that is necessary to sustain epithelial development and morphogenesis (31, 32). As expected, the number of basal cells decreased over time in WT mice, whereas p63 positive basal cell numbers remained elevated through 32 weeks of age in pes-ARKO (SI ZSI Fig. 7*a*). BrdU-positive cells were primarily colocalized with CK5-positive basal cells and to a lesser extent with CK8 positive cells (SI Fig. 6*c*). Localization of cytokeratins-8 and -18 and ZSI pan-cytokeratin-positive luminal cells were similar in WT and pes-ARKO through puberty. However, as pes-ARKO mice aged and AR protein expression decreased, expression of cytokeratins-8, -18 and pan-cytokeratin were diminished (SI Fig. 7*b*). ZSI

Sloughing and Apoptosis of Epithelia in the Prostate of pes-ARKO Mice. To evaluate cell death we used histological analysis and TUNEL staining in VPs from pes-ARKO mice (SI Fig. 6). ZSI

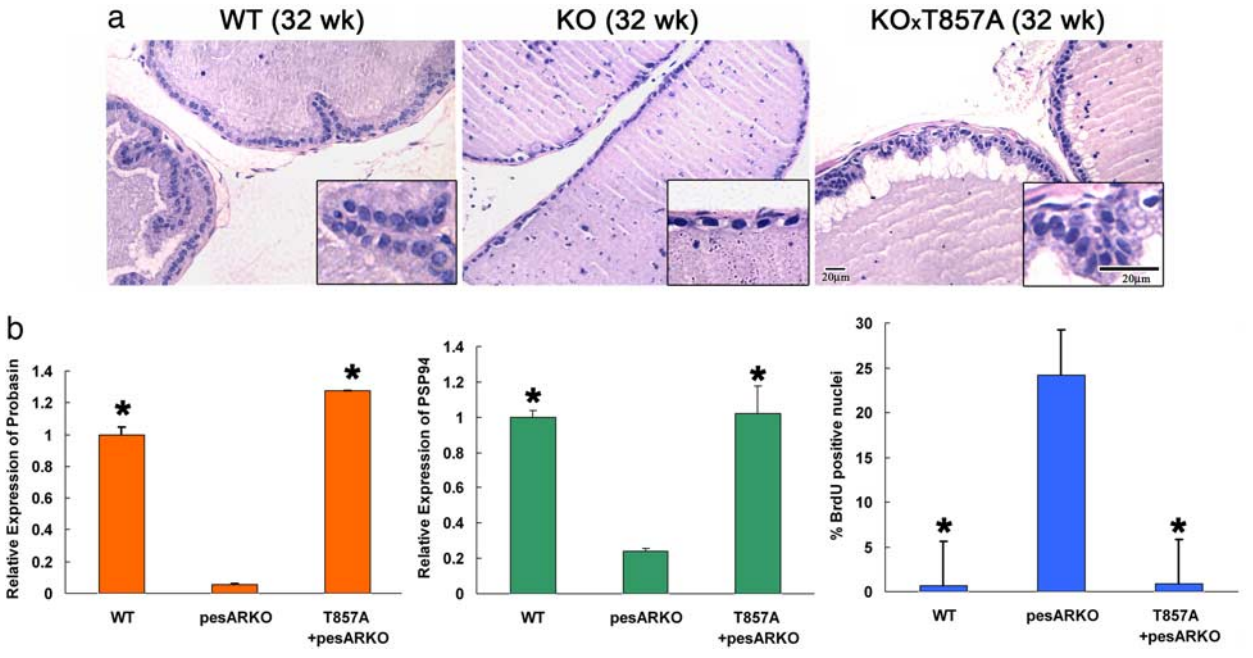

Fig. 4. Expression of T857A mutant androgen receptor transgene in pes-ARKO mice reverts VP phenotype to WT. To determine whether reexpression of AR(T877A) could rescue the pes-ARKO phenotype, we created compound transgenic mice and their prostates were evaluated. (*a*) Hematoxylin and eosin staining of 32 week-old VPs from WT, pes-ARKO, and pes-ARKO/T857A mice. Note that epithelium in pes-ARKO/T857A mice are very similar in morphology, cell height, architecture, and glandular infolding to WT mice. (Magnification: ×100; *Inset*, ×400.) (*b*) pes-ARKO/T857A mice have normal AR gene transcription levels and proliferation rates. Quantitative PCR for probasin (orange bars) expression in VPs at 32 weeks. In pes-ARKO/T857A mice probasin expression is significantly increased compared with pes-ARKO mice, but not different compared with WT. Quantitative PCR for PSP-94 (green bars) expression in VPs at week 32. In pes-ARKO/T857A mice PSP-94 expression is significantly increased compared with pes-ARKO mice, but not different compared with WT. BrdU-labeling index (blue bars) in week 32 VPs. In pes-ARKO/T857A mice epithelial cell proliferation is significantly decreased compared with pes-ARKO mice, but not different compared with WT. *, $P < 0.05$, ***, $P < 0.001$.

However, within the lumen, layers of sloughed epithelium, immune cells, and fragmented DNA were observed. TUNEL analyses showed numerous TUNEL-positive cells or nuclear fragments within the prostatic lumen of pes-ARKO mice. The lack of apoptosis within the epithelial layer was not surprising, because lack of androgen/AR signaling during castration induced prostate apoptosis has been shown to be mediated through the stroma (23). Because there were few apoptotic cells within the intact epithelium, this suggested that TUNEL-positive epithelial cells within the lumen had undergone anoikis (33) by detaching from their basement membrane before the detection of DNA fragmentation, leading to an accumulation of TUNEL-positive DNA and scavenging immune cells within the lumen.

Restoring Functional AR Via Knockin of T857A-AR Restores pes-ARKO to a Normal Prostate Phenotype. Because cellular proliferation and lack of differentiation were observed with removal of WT AR within the prostate epithelia, we wanted to determine whether growth and morphological attributes could be rescued after knockin of constitutively activated T857A-AR (mouse AR mutant equivalent to functional human mutant AR, T877A), found in LNCaP cells and human prostate tumors (34, 35) into prostate epithelia of pes-ARKO mice. Therefore, we created triple-mutant mice containing T857A-AR, flox-AR, and ARRPB2-cre, resulting in the T857A/pes-ARKO mice, which have no WT AR within the prostate epithelium but express transgenic T857A-AR. Genomic DNA (PCR), mRNA (RT-PCR), and protein assays (immunohistochemistry) all demonstrated that deletion of WT AR and appropriate expression of T857A-AR in T857A/pes-ARKO mice occurred (data not shown). We saw no external differences between T857A/pes-ARKO and WT or pes-ARKO mice. Gross dissection of T857A/pes-ARKO mice revealed little differences in internal urogenital organs between WT and pes-ARKO mice at any stage. Importantly, VPs were similar in size compared with WT littermates. VPs were much smaller in T857A/pes-ARKO mice than in pes-ARKO mice. As anticipated, restoring AR signaling in pes-ARKO mice generated a normal glandular epithelial phenotype quite similar to that of WT mice at week 32 (Fig. 4). This included the presence of glandular infolding and tall secretory columnar cells. In addition to restoring normal prostate architecture, the expression of functional AR within the epithelia of T857A/pes-ARKO mice stimulated biochemical changes and reexpression of differentiation markers within the epithelium. These changes included increased gene expression of secretory proteins PSP94 and probasin (Fig. 4*b*). To measure epithelial cell proliferation in T857A/pes-ARKO mice, we determined the BrdU labeling index as described above. Importantly, VPs in T857A/pes-ARKO mice were smaller than those in pes-ARKO mice, suggesting a lack of proliferation like that in prostate from WT mice. The restoration of androgen/AR action within pes-ARKO mice significantly reduced epithelial proliferation to levels not different from WT littermates (Fig. 4*b Right*). Collectively, these gain-of-function experiments show that AR can suppress prostate epithelial proliferation both *in situ* and *in vivo*, a role that has previously been ascribed to stromal factors. However, in these studies implicating stromal factors, tissues were evaluated at 1–2 months, whereas our studies implicating epithelial AR exhibited such effects at >6 months (5, 6).

Conclusion

A key signature of the adult normal prostate gland is the lack of proliferation even in the presence of growth stimulating androgens. This is in contrast to benign prostate hyperplasia and prostate cancer, in which epithelial cells acquire the ability to proliferate. Here, we show two seminal findings in the area of cell

biology. First, we report *in vivo* and *in situ* that in mature prostatic epithelium, AR is critical for maintaining the differentiated phenotype and overall homeostasis of the gland. Moreover, selective removal of AR signaling in luminal and basal epithelial cells stimulates mitogenesis of the otherwise growth quiescent adult prostate. These data support the hypothesis that epithelial AR maintains homeostasis via induction of epithelial proliferation suppressors or through the decreased production of proliferation-stimulatory factors. These proliferation regulators may indirectly mediate stromal factors or act directly on the putative AR-negative progenitor (i.e., transit amplifying or intermediate) cells, thereby inhibiting epithelial proliferation. The mechanisms by which AR mediates these processes might be multifactorial, most likely involving paracrine factors. Normal prostate growth might require delicate temporal and spatial balance between the proliferative role of stromal AR and the proliferation-suppressive role of epithelial AR. Our findings recast the role of androgen/AR signaling within the prostate and suggest that future chemoprevention strategies might need to target stromal-AR-mediated-factors rather that epithelial-AR-mediated-factors to prevent early stages of carcinogenesis.

AQ: I

Methods

Generation of Transgenic Mice. To generate pes-ARKO mice, we mated ARRPB2-Cre transgenic mice (13) (C57BL/6N, from NIH) with mice (C57BL/6J) containing the conditional AR allele (floxed AR; SI Fig. 8) (3). To generate pes-ARKO/T857A AR mice, we interbred the three transgenic mice, ARRPB2-Cre mice (C57BL/6N), floxed AR mice (C57BL/6J), and T857A AR mice (FVB) (gift from Dr. N. Greenburg, Fred Hutchinson Cancer Research Center, Seattle, WA).

ZSI

AQ: J

Immunohistochemistry. Pes-ARKO specimens: All three lobes were embedded in the same block and sections prepared at 5 μm. Immunodetection was performed as described in refs. 36 and 37. The antibodies used were: anti-AR (C-19, 1:200), anti-probasin (1:300), anti-CK8/18 or anti-probasin *R-15* (1:100), anti-PCNA (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-p63 (1:50) (abcam). The ratio of AR-positive to total nuclei was calculated in at least 500 cells examined in each of three randomly selected regions.

RNA Isolation and Analysis. Total cellular RNA was isolated from each lobe and used to synthesize random primed first-strand complementary DNA for analysis by RT-PCR or real-time PCR (36). Amplification of AR exon 2, Nkx3.1, probasin, and PSP 94 were normalized to beta-actin in each sample. Sequences used were as follows: Probasin, 5′-ATC ATC CTT CTG CTC ACA CTG CAT G-3′ (forward), 5′-ACA GTT GTC CGT GTC CAT GAT ACG C-3′ (reverse); PSP-94, 5′-CCT GTA AGG AGT CCT GCT TTG TC-3′ (forward), 5′-ATG CTG GCT CTG CCT TCT GAG T-3′ (reverse); Nkx3.1, 5′-AGA CAC GCA CTG AAC CCG AGT CTG ATG CAC-3′ (forward), 5′-AGA CAG TAC AGG TAG GGG TAG TAG GGA TAG C-3′ (reverse).

AQ: K

Apoptosis Assay. The *in situ* cell death detection kit (Roche Pharmaceuticals, Nutley, NJ) was used according to the manufacturer's instructions for detection of apoptotic cells.

BrdU Labeling Indices. Mice were injected with BrdU (30 μg/gm body weight; Sigma–Aldrich, St. Louis, MO) i.p. 24 h before killing. The BrdU-labeled epithelial cells were detected employing a monoclonal anti-BrdU antibody Zymed Laboratories, San Francisco, CA) according to manufacturer's direction. The labeled cells were calculated from multiple fields of each slide. Several sections from each prostate were analyzed to obtain the mean of BrdU positive epithelial cells. The means of the proliferating cells from each lobe of prostate were reported.

AQ: L

Other Methods. We performed testosterone quantification, PCR, and Western blot assays as described in ref. 36.

Statistics. We presented the data as the mean ± SD. We made comparisons between groups, using a two-sided Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered significant.

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